CHROMATOGRAPHY OF NUCLEIC ACIDS ON HYDROXYAPATITE

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CHROMATOGRAPHY on hydroxyapatite was introduced as a very useful tool in protein chemistry by Tiselius, Levin and Hjertén[1]. In my experience, this technique is particularly advantageous in the separation of phosphoproteins[2] and of proteins of very close charge and/or size[3], as well as in the purification of virus particles, like T2 bacteriophage, tobacco mosaic virus and turnip yellow mosaic virus[4].

Results obtained in this laboratory during the past five years in the area of polynucleotides are presented here. Some preliminary reports have already been published[6-9]. The chromatographic experiments were performed at room temperature on hydroxyapatite prepared according to Tiselius et al.[1].

Chromatography of native DNA. Chromatographic fractionations of DNA on hydroxyapatite were reported by Semenza[10] and by Main et al.[11,12]; but their meaning was obscure because of the lack of unequivocal re-chromatography experiments and of adequate physical characterization of the starting DNA and the fractions; furthermore, no fractionation was achieved on the basis of DNA composition.

Since it had not been established whether any changes occurred in the DNA properties on the adsorption-desorption process, this point was examined first. DNA samples were adsorbed on hydroxyapatite equilibrated with 0·005 M phosphate buffer pH 6·8. Up to 2 per cent of (acid-soluble) material was not retained by the column in some samples. The adsorbed material was then completely eluted with 0·5 M phosphate buffer pH 6·8. The eluted product displayed no significant differences with respect to the loaded DNA, as judged by light-scattering, sedimentation, ultra-violet absorption and 'melting curve'. This behaviour was consistently found with many DNA samples from chicken erythrocytes, calf thymus and *E. coli* showing molecular weights in the 4–6 × 10⁶ range. The recovery from the columns of the much larger DNA molecules from phages T2 and T5 was also complete. Preliminary results with T2 DNA 'whole' molecules indicated that the sedimentation constants of the loaded and eluted DNA were identical. *H. influenzae* DNA was adsorbed and eluted from hydroxyapatite without any
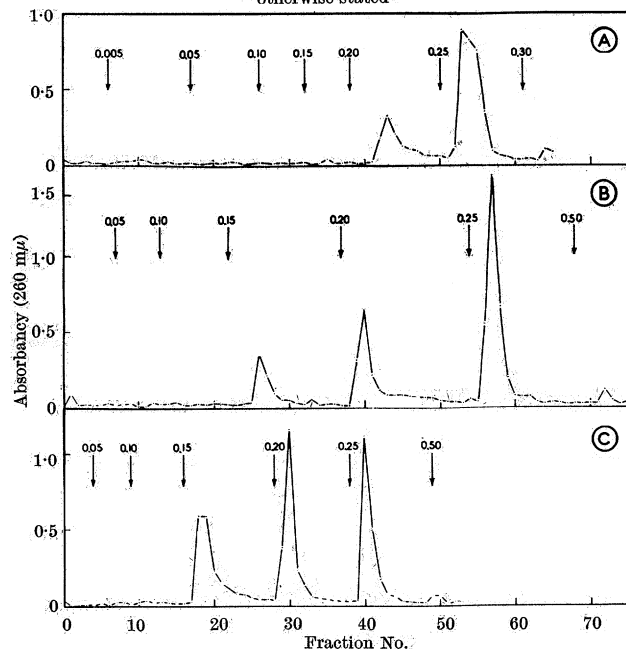
Fig. 1. Stepwise elution of: A, native DNA; B, heat-denatured DNA (90°); C, heat-denatured DNA (100°); recovery, 95 per cent

modification to its transforming ability for three different genetic markers[13].

The second point investigated concerned the possibility of fractionating native DNA. All DNA preparations from chicken erythrocytes, calf thymus and E. coli tested so far were eluted at 0·20 and 0·25 M phosphate, when elution was performed stepwise as in Fig. 1A. Occasionally, minor additional fractions were eluted when the phosphate molarity was raised to 0·30 and 0·50 M. On rechromatography all these different fractions were again eluted in two peaks, at 0·20 and 0·25 M phosphate, respectively. The peaks obtained appear therefore to be 'false' peaks, a type of artefact already examined by Tiselius et al.[1] and Hjertén[14]. In agreement with this conclusion is the finding that when elution was performed with a linear molarity gradient, all native DNA samples investigated so far were eluted in one peak (Fig. 3A).

A very limited fractionation according to molecular weight does, however, take place on hydroxyapatite, as
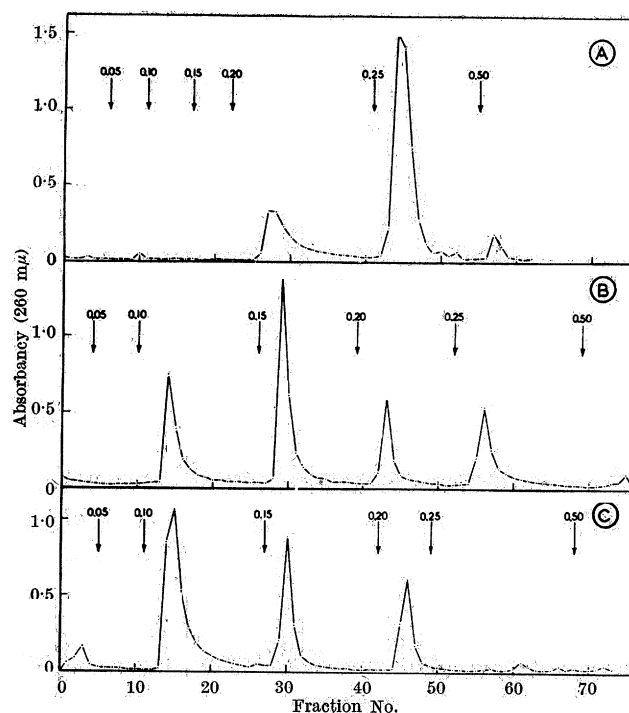
Fig. 2. (See also legend to Fig. 1.) Stepwise elution, in the presence of 1 per cent formaldehyde, of: *A*, native DNA; *B*, heat-denatured DNA (100°); *C*, heat-denatured DNA (100°, in the presence of formaldehyde); recovery, 93 per cent

suggested, for example, by the slightly lower molecular weight of the lower eluting fractions, and by the separation of 'cold' *T*2 DNA 'whole' molecules (mol. wt. ~ 1.3×10^8) from sonicated phosphorus-32-labelled *T*2 DNA (mol. wt. ~ 5×10^5). It is doubtful, however, whether these fractionations are due to the chromatographic process itself, since the elution curves of DNA samples ranging in molecular weight from 6×10^6 to 1×10^5 were essentially identical. These samples were obtained by limited digestion with acid deoxyribonuclease, an enzyme able to cut across the two strands at the same level and which does not alter significantly the native double-stranded structure until molecular weights of the order of 10^5 are reached[15]. On the other hand, DNA samples enzymatically degraded to molecular weights lower than 5×10^4 showed fractions eluting at lower phosphate molarities than intact samples. Fractions eluted at 0.05 M phosphate or lower appeared to be formed by oligonucleotides of different sizes. Fractions eluted at 0.10 and 0.15 M phosphate had

a larger molecular weight and a considerable amount of secondary structure since they showed a hyperchromicity of 15–18 per cent on alkalinization.

Chromatography of heat-denatured DNA. Heat-denaturation was performed by heating at 100° for 15 min chicken erythrocytes or calf thymus DNA solutions at a concentration of 50–100 µg/ml. in 0·13 M sodium chloride–0·01 M phosphate buffer pH 6·8; these were then rapidly cooled in an ice-bath. The DNA solutions were chromatographed either as such, or after having been reacted at 25° for 24 h with formaldehyde (final concentration 1 per cent); in the latter case the eluting buffers contained 1 per cent formaldehyde. Heat-denatured formaldehyde-reacted DNA was mainly eluted at 0·15 M phosphate, with smaller fractions at 0·10, 0·20, 0·25 M and sometimes a minor one at 0·50 M phosphate (Fig. 2B), a pattern remarkably different from that of native DNA run in the same experimental conditions (Fig. 2A).

The chromatographic fractions obtained from heat-denatured, formaldehyde-reacted DNA were dialysed against 0·13 M sodium chloride–0·01 M phosphate buffer pH 6·8 containing formaldehyde (1 per cent final concentration) and heated for 15 min at 100°. The increase in optical density at 260 mµ obtained on this treatment was used as a measure of the extent of native-like double-stranded structure of the fractions (see following). This increase was found to be extremely small (0·5 per cent) in the 0·10 and 0·15 M fractions, while the 0·25 M fraction (and the 0·50 M fraction, if present) showed an increase of about 15 per cent. The starting, native DNA showed, on the same treatment, a hyperchromic shift of 38–40 per cent, and the heat-denatured, unfractionated DNA showed an increase of about 5 per cent. Furthermore, the 0·25 M fraction, in contrast to the 0·10 and 0·15 M fractions, showed a sharp melting curve.

These findings and the re-chromatography experiments (not shown here) indicated that two different fractions were present. When gradient elution was used (Fig. 3B), heat-denatured, formaldehyde-reacted DNA was eluted in one main peak at about 0·12 M phosphate, followed by a minor fraction at about 0·20 M. These fractions were equivalent to the 0·10–0·15 M fractions and to the 0·25–0·50 M fractions of the stepwise elution, respectively. Artificial mixtures of native and heat-denatured DNA were easily separated on hydroxyapatite (Fig. 3C). The behaviour of DNA which had been heat-denatured, but not reacted with formaldehyde, was comparable to that just described with the difference that the lower eluting fractions were less important, so that the whole chromatogram was shifted to the right (compare Fig. 1C with Fig. 2B). When heat-denatured *H. influenzae* DNA was run on hydroxyapatite using a gradient elution, most of the residual transforming activity was found in the small peak of partially denatured
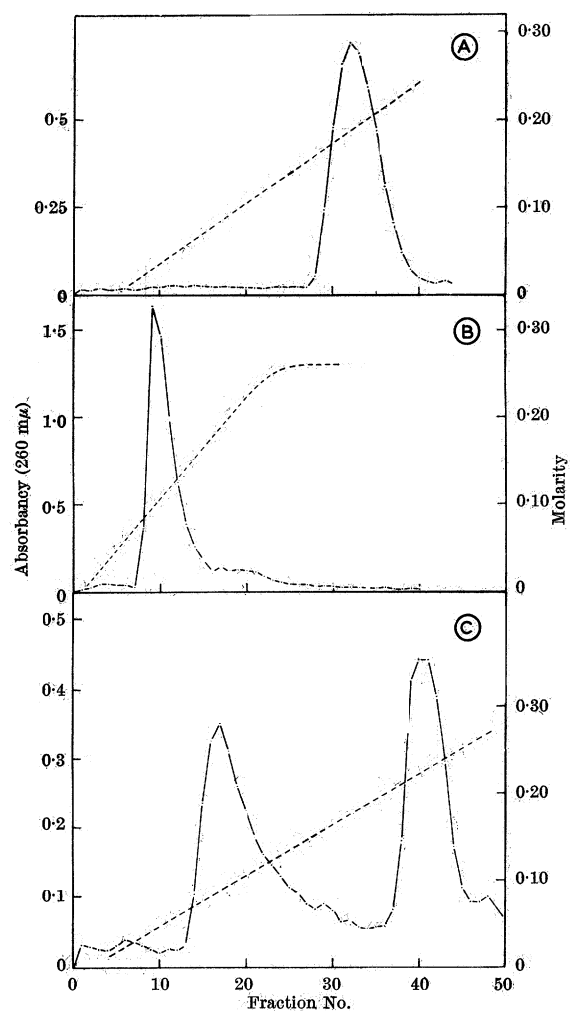
Fig. 3. (See also legend to Fig. 1.) Gradient elution of: *A*, native DNA; gradient elution, in the presence of 1 per cent formaldehyde; *B*, heat-denatured DNA (100°); recovery, 95 per cent; *C*, a 1 : 1 mixture of native and heat-denatured (100°) DNA

molecules[13]. The single-stranded DNA from phage φX 174 was eluted at 0·15 M phosphate with a smaller fraction at 0·10 M.

Finally, DNA samples which were denatured in the presence of 1 per cent formaldehyde, in which condition the melting temperature is lowered by 10°–15°, and chromatographed in the presence of formaldehyde, showed, in contrast to the previous case, an elution

profile (Fig. 2C) significantly shifted to the left, and extremely little material was eluted at molarities higher than 0·20.

Fractionation of DNA molecules according to their average base composition. The experiments with heat-denatured DNA, just reported, indicate that hydroxyapatite fractionates DNA molecules according to their secondary structure since the percentage of native-like structure of the fractions was regularly increasing with the increase of their eluting molarities. If the native-like regions were due to an intramolecular process of re-annealing around GC-rich 'nuclei', as in type I reversibility of Geiduschek[16], one would expect that GC-rich molecules would show a larger amount of native-like regions compared to the GC-poor molecules, and elute at higher molarities than the latter. This is exactly what one actually finds. For example, in the case of calf thymus DNA heated to 100° and reacted with formaldehyde (Fig. 2B), the A + T/G + C ratio of the different fractions was found to be 1·58 for the 0·10 M, 1·26 for the 0·15 and 0·95 for the 0·25 M fraction, the ratio of the starting sample being 1·29–1·28.

The fractionation of DNA molecules according to their average base composition may be improved by using different denaturation temperatures; in fact, by changing the denaturation temperature it is possible to change at will the relative proportions of the various fractions (compare Figs. 1B and 1C).

The procedure developed three years ago[3] involved heating aliquots of a given DNA sample up to various temperatures and fractionating them on columns. Other experimental conditions may lead to similar results, however. For example, chromatographic experiments may be performed at different high temperatures and constant ionic strength (see following); this permits a separation of melted from unmelted molecules at the temperature considered.

Chromatography of RNA and polyribonucleotides. High-molecular-weight RNA from Ehrlich ascites tumour cells[7], tobacco mosaic virus, turnip yellow mosaic virus, are all eluted in two peaks at 0·15 and 0·20 M phosphate (Fig. 4), when using a stepwise elution. They thus display a chromatographic behaviour similar to that of heat-denatured DNA, with the remarkable difference that no fraction is eluted at molarities higher than 0·20 M. Soluble RNA from *E. coli* or yeast was eluted at about 0·13 M phosphate, when using gradient elution (Fig. 5A). RNA 'core', the undialysable residue left after digestion of RNA by pancreatic RNase, was eluted at 0·015 M phosphate (Fig. 5B), therefore at the same molarity level as DNA oligonucleotides (see foregoing).

Synthetic polyribonucleotides (purchased from Miles or Calbiochem) were also investigated. Polyuridylic acid, a polymer devoid of any organized secondary structure under the conditions used in the chromatography, was
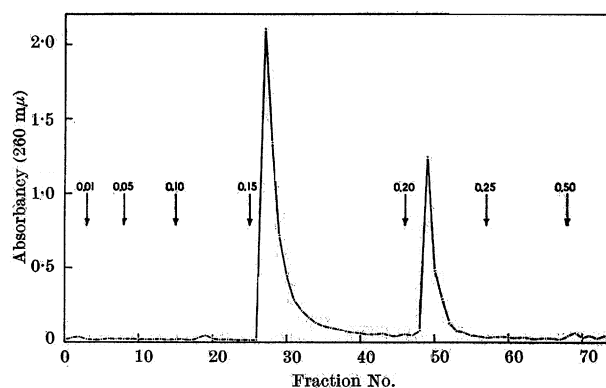
Fig. 4. Stepwise elution of high-molecular-weight RNA from Ehrlich ascites tumour cells. Loading at fraction number zero. 3·8-ml. fractions were collected. Recovery was 100 per cent

eluted at 0·10 M phosphate (Fig. 6A). On the other hand, polyadenylic acid, which has some helical structure under the experimental conditions used, was eluted in a broad peak at 0·20 M phosphate; a considerable amount of low-molecular-weight material was eluted at a very low molarity (Fig. 6B). The artificial complexes of poly U and poly A were also examined, under conditions where double-stranded poly A–poly U or triple-stranded 2 poly U–poly A were formed. The double-stranded poly A–poly U complex was eluted at a molarity only slightly
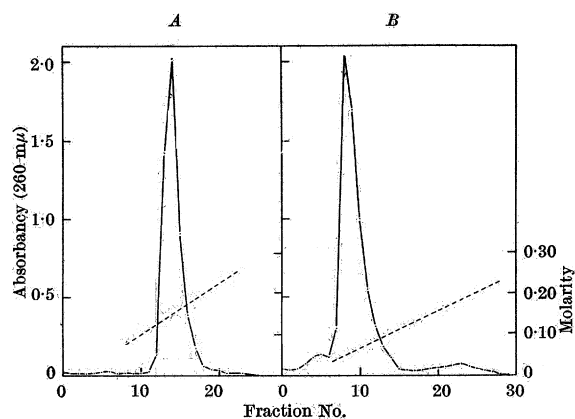

Fig. 5. Chromatography of: A, yeast soluble RNA; B, yeast RNA 'core' on 1 cm. × 10 cm. hydroxyapatite columns; loading at fraction number zero. 3 ml. fractions were collected. Recoveries were 97 and 96 per cent, respectively
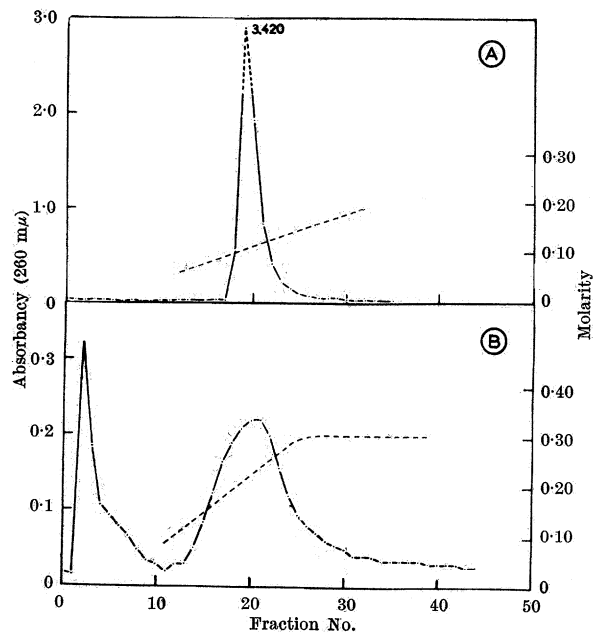
Fig. 6. Chromatography of: *A*, polyuridylic acid; *B*, polyadenylic acid on 1 cm × 10 cm hydroxyapatite columns. 3 ml. fractions were collected. Recoveries were 94 and 75 per cent, respectively

higher than poly A; the low eluting material was not any more apparent in this case (Fig. 7*B*). The triple-stranded complex was eluted at a much higher molarity, in the 0·45–0·50 M phosphate region (Fig. 7*A*).

Adsorption mechanism. It is likely that the adsorption of polynucleotides on hydroxyapatite takes place because of the electrostatic interaction between the negative phosphate groups of the nucleic acids and the positive calcium ions of the hydroxyapatite crystals. Increasing the molarity of the eluting phosphate buffer progressively reduces this interaction to zero, at which point desorption occurs. The decrease in the interaction appears to be due to a specific competition between the phosphate ions of the eluting buffer and the phosphates of polynucleotides for the calcium ions of hydroxyapatite, and not simply to increase of ionic strength. In fact, if elution is performed at constant ionic strength, with a linear gradient between 1 M potassium chloride + 0·001 M potassium phosphate buffer *p*H 6·8 and 0·5 M potassium phosphate buffer *p*H 6·8, native DNA is desorbed essentially at the same phosphate molarity as in the absence of potassium chloride.

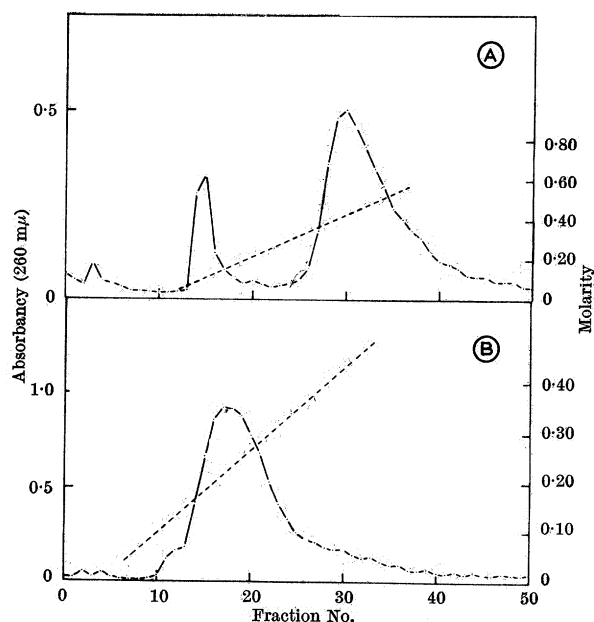The foregoing explanation is also supported by the finding that treatment of hydroxyapatite with dilute

Fig. 7. Chromatography of: A, polyuridylic acid + polyadenylic acid
(3 : 1); B, polyuridylic acid + polyadenylic acid (1 : 3) on 1 cm × 10 cm
hydroxyapatite columns. 3-ml. fractions were collected. Recoveries
were 60 and 95 per cent, respectively

chelating agents reduces the adsorption capacity of
hydroxyapatite for polynucleotides, an observation
already made for proteins by Tiselius et al.[1]. Furthermore,
an investigation on nucleoside mono- and poly-phosphates
and phosphorylated coenzyme derivatives[9] showed that
their adsorption on hydroxyapatite is only related to the
ionization of the phosphate groups, with no interference
by the organic moieties of the molecules.

Taking into account the fact that large polynucleotides
may be considered of infinite length for the present pur-
pose, it may be expected that the only property respon-
sible for their chromatographic behaviour will be their
linear charge density. In fact, the finding that heat-
denatured DNA has a lower affinity for hydroxyapatite
than native DNA is in agreement with the reports that
the charge density of denatured DNA[17] and its electro-
phoretic mobility[18,19] are lower than those of native DNA.
The chromatographic results obtained with RNAs and
synthetic polyribonucleotides and the effect of form-
aldehyde on the elution of heat-denatured DNA provide
further support to the interpretation given here.

Conclusion. The results reported here establish chrom-
atography on hydroxyapatite as a new, powerful tech-
nique in the field of nucleic acids. Among its advantages

over other already existing techniques, three are of special importance. The first is that the chromatographic substrate is an inorganic, crystalline, insoluble salt; it is therefore possible to work over a wide range of temperatures, as well as in the presence of organic reagents, like formaldehyde, phenol, chloroform, etc. The second is that the adsorption–elution phenomenon is fundamentally associated to one single property of the polynucleotides, namely, their linear charge density; this makes hydroxyapatite chromatography a process understandable in terms of known physicochemical laws. The third point is that this technique is simple, easy and reproducible and does not require any expensive equipment or reagent.

Basically, hydroxyapatite lends itself to the fractionation and characterization of polynucleotides as indicated by the examples presented here. Another use which may be briefly mentioned here is related to the preparation of nucleic acids, particularly DNA or RNA of viral origin. In this latter case hydroxyapatite may be used, in columns and in batches as well, both to concentrate and purify the virus particles and to separate nucleic acids from the phenol-treated virus. Several possible applications of this technique will be discussed elsewhere, in a detailed presentation of this work.

[1] Tiselius, A., Levin, O., and Hjertén, S., *Arch. Biochem. Biophys.*, **65**, 132 (1956).

[2] Bernardi, G., and Cook, W. H., *Biochim. Biophys. Acta*, **44**, 86 (1960).

[3] Bernardi, G., Bernardi, A., and Chersi, A. (in preparation).

[4] Bernardi, G. (in preparation).

[5] Bernardi, G., Comm. at First Intern. Symp. Chromatography, Brussels, September (1960).

[6] Bernardi, G., *Biochem. Biophys. Res. Comm.*, **6**, 54 (1961).

[7] Bernardi, G., and Timasheff, S. N., *Biochem. Biophys. Res. Comm.*, **6**, 58 (1961).

[8] Bernardi, G., *Biochem. J.*, **83**, 32P (1962).

[9] Bernardi, G., *Biochim. Biophys. Acta*, **91**, 686 (1964).

[10] Semenza, G., *Arkiv Kemi*, **11**, 89 (1957).

[11] Main, R., and Cole, L. J., *Arch. Biochem. Biophys.*, **68**, 181 (1957).

[12] Main, R., Wilkins, M. J., and Cole, L. J., *J. Amer. Chem. Soc.*, **81**, 6490 (1959).

[13] Chevallier, M.-R., and Bernardi, G., *J. Mol. Biol.*, **11**, 658 (1965).

[14] Hjertén, S., *Biochim. Biophys. Acta*, **31**, 216 (1959).

[15] Bernardi, G., and Sadron, C., *Biochem.*, **3**, 1411 (1964).

[16] Geiduschek, E. P., *J. Mol. Biol.*, **4**, 467 (1962).

[17] Ascoli, F., Botré, C., and Liquori, A. M., *J. Mol. Biol.*, **3**, 202 (1961).

[18] Matsubara, K., and Takagi, J., *Biochim. Biophys. Acta*, **55**, 389 (1962).

[19] Costantino, L., Liquori, A. M., and Vitagliano, V., *Biopolymers*, **2**, 1 (1964).